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INVESTIGATIONS INTO THE EFFICIENCY OF DIALDEHYDES AND OTHER COMPOUNDS AS CROSS-LINKING AGENTS FOR COLLAGEN

Part II

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Summary

A number of bi- and poly-functional compounds comprising the linear dialdehydes with up to six C-atoms, tetrakis (hydroxymethyl) phosphonium chloride, dialdehyde starch, two di-isocyanates and a number of di-isocyanate generators and several chlorotriazine derivatives are assessed as cross-linking agents for collagen. Treatments are mainly on kangaroo tail tendon and the number and relative stabilities of the cross-links are assessed by stress-strain analysis after hydrothermal denaturation of the specimens.

All the compounds listed caused cross-linking in some degree. Aldehydes of more than one function offer the greatest promise. The simpler chlorotriazines are limited by low solubility in aqueous systems and the class as a whole by the relatively low reactivity of the final chlorine atom to be substituted in cross-link formation. The di-isocyanates with their predilection to polymerisation are not attractive and their addition compounds do not introduce an effective number of cross-links.

Introduction

A method of estimating the number of covalent cross-links introduced between the polypeptide chains of collagen by reaction with bifunctional compounds has been described in Part I of this series*. Experimentally the method consisted of stress-strain and volume fraction measurements on hydrothermally denatured tendon and relied theoretically on the Flory-Rehner equation—an equation originally developed for a swollen elastomer. Applicability of the method hinged, therefore, upon similarity of properties between rubber and chemically modified collagen. Hydrothermally denatured collagens, equilibrated in water at 65°c, were found to closely approximate to rubber-like behaviour.

In the present paper study of the action on collagen of the first five dialdehydes from glyoxal, where the two CHO-groups are directly linked, to adipaldehyde where they are separated by four methylene groups, is completed. Also reported are the actions of other bi- and poly-functional compounds such as dialdehyde starch, tetrakis (hydroxymethyl) phosphonium chloride, the di-isocyanates and their addition compounds and cyanuric chloride together with other related symmetrical triazine derivatives. In most cases determinations have been made of the number of cross-links initially introduced and of those remaining after the application of a specific treatment designed to test the stability of the bonds involved.

Experimental

Determination of the number of cross-links introduced into kangaroo tail tendon was by methods essentially similar to those already described with the following modifications.

Originally the determination of the length at zero stress relied heavily upon the operator's judgment. Improved reproducibility has been obtained by adopting the following procedure: The equilibrated relaxed specimen was straightened by moving the lower extensiometer clamp downwards until it was almost in tension. The approximate length of the specimen could then be obtained from the calibrated scales on the screw-stand and, knowing this, a suitable extension step calculated. The specimen was extended at intervals of two minutes until loads corresponding to five or six steps had been recorded on a graph of load versus extension. The almost linear curve was extrapolated to zero load and the intercept on the extension axis was added to the initial distance between the clamps to give the unstressed length of the specimen.

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Once the number of steps necessary to establish the unstressed length had been performed it was found permissible to complete the curve by extending directly to the maximum, i.e. 16 per cent of unstressed length without loss of accuracy. The steady final value for load was normally attained within four minutes of increasing the length to maximum.

(i) Sources of Materials

(*a*) *Kangaroo Tail Tendons.* The Zoological Society of London, the Defence Standards Laboratory, Maribyrnong, the Wool Research Laboratories, New South Wales, and the Research Station of the Department of Agriculture and Stock, Warwick, Queensland, kindly supplied the tails from which the tendons used in Parts I and II of this work were extracted. Staffs of the three Australian laboratories also co-operated in preparation of the tendons by the method given below. Air-dry surgical suture kangaroo tail tendon (used in Part I) was a gift from Ethicon Inc.

The tendons were dissected out from fresh kangaroo and wallaby tails and immediately placed in a large volume of 5% $^{w}/_{v}$ sodium chloride solution. Over the next 24 hours they were soaked in three changes of fresh salt solution and then thoroughly washed in running water. After draining well they were dehydrated in several changes of acetone and finally soxhlet extracted with ether. Stocks of material were stored air-dry until required.

(*b*) *Chemicals*. Malonaldehyde was generated from its tetraethyl acetal (Aldrich Chemical Company) by the action of $N.H_2SO_4$. Succinaldehyde was similarly obtained from diethoxytetrahydrofuran (Koch-Light Laboratories Ltd.). Glutaraldehyde was obtained as a 25% solution in water (Union Carbide Corporation). Adipaldehyde was synthesised by the following route: cyclohexanone$\rightarrow$ cyclohexanol$\rightarrow$ cyclohexene$\rightarrow$ cyclohexane diol$\rightarrow$ adipaldehyde. The methods used were based on a synthesis of glutaraldehyde previously carried out in this laboratory to prepare the ^{14}C labelled material[1]. Dialdehyde starch (93% oxidised) (hereafter DAS), a gift from the Eastern Regional Laboratories of the U.S. Department of Agriculture, was dissolved as follows: DAS (25 g.) was stirred into a solution of borax (1.4 g.) and water (75 ml.). The suspension with continued stirring, was heated on a steam bath. The pasty mass dissolved almost completely on maintaining at approximately 80° for about an hour. This solution was suitably diluted before use.

1:6-hexamethylene di-isocyanate (HDI) and 2:4-toluene di-isocyanate (TDI) were obtained from Koch-Light Laboratories Ltd. The di-isocyanate bisulphite addition compounds were prepared as follows: sulphur dioxide was passed into a cooled aqueous solution of potassium hydroxide (40% $^{w}/_{v}$) until saturated with the gas. A small quantity of HDI or TDI was then added so that the bisulphite was much in excess. On shaking the adduct separated as a white or pale yellow solid which was filtered off via a sintered glass funnel, washed with a little cold water and vacuum dried[2].

The succinimide addition compound with HDI:- HDI (1 mole) was added to a saturated solution of the imide (2 mole) in water, with cooling. After shaking intermittently, a white solid separated and was filtered, washed and dried as above[2].

Cyanuric chloride (British Drug Houses Ltd.) was purified by dissolving in carbon tetrachloride, filtering off the insolubles and evaporating the filtrate to dryness.

Sodium (dichloro-s-triazine)-1-naphthalide-5 sulphonate and " Procion " dye Yellow M-RS were gifts from Imperial Chemical Industries Ltd.* 2-amino-4:6-dichloro-s-triazine hydrochloride was prepared from cyanuric chloride by the method of Diels[3] and sodium dichloro-s-triazine sulphonate was prepared by a method based on the work of Atkinson, Hall and Stephen[4]. Disodium-di (dichloro-s-triazine)-4:4′-diaminostilbene-2:2′-disulphonate was prepared from 4:4′-diaminestilbene-2:2′-disulphonic acid (Koch-Light Laboratories) and cyanuric chloride by a method modelled on that of Adams and Wilson[5]. 2-Methoxy-4:6-dichloro-s-triazine was prepared by the method of Dudley *et al*[6].

Tetrakis (hydroxymethyl) phosphonium chloride (THPC) was a gift from Albright and Wilson Ltd.

(ii) Treatment of Tendon and Limed Skin Pieces

Except as otherwise stated below, all chemical treatment of skin and tendon were preceded by soaking in water followed by equilibration in saline (either 4% $^w/_v$ sodium chloride or 10% $^w/_v$ sodium sulphate) at pH 3·0, twelve volumes of saline to one part by weight of dry collagen being normally used. Treatments were carried out in stoppered bottles at room temperature with intermittent agitation. After addition of the reagent the pH's were maintained at 3·0 for an hour to allow penetration of the samples without appreciable reaction. The pH's were then raised and maintained virtually constant by addition of suitable buffer salts. Afterwards the samples were washed, equilibrated to pH 5·0, washed again and finally dehydrated in three changes of acetone.

Dialdehyde and THPC treatments were for 16 hours. In the case of DAS the time was extended up to 72 hours.

Since HDI is insoluble in water an emulsifying agent was added ("Teepol" 610 or "Nonidet" P40, Shell Chemical Company). Polymerisation occurred in the emulsion after about 2 hours; treatment was discontinued at this point. The di-isocyanate addition compounds were made up into saturated solutions and used in pH range 5-6 at temperatures ranging from 20° – 30°. HDI and TDI treatments were also carried out in organic solvents. In Pyridine the procedure was:— HDI (1 part) or TDI (1 part) was dissolved in dry pyridine (20 parts). The collagen was introduced in the air-dry condition and treatments were in the temperature range 20° – 50°, with the di-isocyanate in large excess. After treatment the collagen was washed in three changes of pyridine and dried under vacuum.

Using benzene as solvent, the presence of a small amount of tertiary amine was found essential for reaction *e.g.* using the proportions above, triethylamine (1 part) was added.

* The word " Procion " is a registered Trade Mark of Imperial Chemical Industries Ltd.

Cyanuric chloride and 2-methoxy-4:6-dichloro-s-triazine are only slightly soluble in water. They were, therefore, dissolved in six volumes of acetone. The collagen was soaked out in water and then transferred into six volumes of 5% $^{w}/_{v}$ sodium bicarbonate solution. The solution of the triazine was then added with stirring.

(iii) Stability Tests

(*a*) *Stability to boiling water.* The tendon was suspended in about 500 mls of boiling water for the time stated before cross-links were determined.

(*b*) *Stability to acid.* The tendon was treated in $N.H_2SO_4$ at 20° for the stated period, then washed in water, equilibrated in pH 5 buffer solution and thoroughly washed again. Before testing it was denatured for the standard time of two minutes in boiling water.

(*c*) *Stability to perspiration.* The perspiration solution was made up to the following composition:—

sodium lactate (100%)	5·0 g
sodium chloride	5·0 g
glycine	0·5 g
*" Nipasept "	0·1 g
water	100 ml

* An antiseptic made by Nipa Laboratories Ltd., London. It is composed of esters of p-hydroxy benzoic acid.

The natural pH of this solution was 6·76. A second solution at pH 7·70 was prepared by incorporating 1·3 g per 100 ml sodium bicarbonate into the solution.

Tendons were first soaked out well in water, blotted and then submerged in the perspiration solution (10 parts of solution to one of air-dry tendon) for 24 hours at 40°. They were then sealed in polythene bags and placed in an incubator at 40° for periods up to 28 days. On removal from the bags they were air-dried.

(*d*) *Stability to saturated lime.* The tendon was soaked in water, blotted and placed in saturated lime (10 parts of solution to one of air-dry tendon) in a stoppered flask which was incubated at 40°.

Results

Stress-strain results may be expressed in two ways. The usual form of the Flory-Rehner equation permits calculation of the mean molecular weight per cross-linked chain segment, denoted by Mc. To convert Mc to moles of cross-links per given mass of material, the number of chain segments which meet in each cross-link must be postulated and the present series of results are calculated on the basis of four chains. The number of cross-links per 10^5g is then given by $10^5/2M_c$.

(i) Dialdehydes

The numbers of cross-links introduced into collagen by glyoxal and glutaraldehyde using a substantial excess above that required to satisfy all potentially reactive groups, were determined in Part I of this series. These

TABLE I

Cross-Links Introduced by Dialdehydes in Excess

Aldehyde	pH of treatment	M_c	Number of cross links per 10^5g
*Glyoxal	8·0	5,900	8
Malonaldehyde	5·0	12,600	4
	6·0	9,500	5
Succinaldehyde	6·0	5,800	9
	7·0	5,100	10
*Glutaraldehyde	4·0	7,000	7
	6·5	3,900	13
	8·0	5,600	9
Adipaldehyde	6·5	24,000	2

M_c = the mean molecular weight per cross-linked chain segment.

* The results for glyoxal and glutaraldehyde were reported in Part I. They are repeated to complete the series.

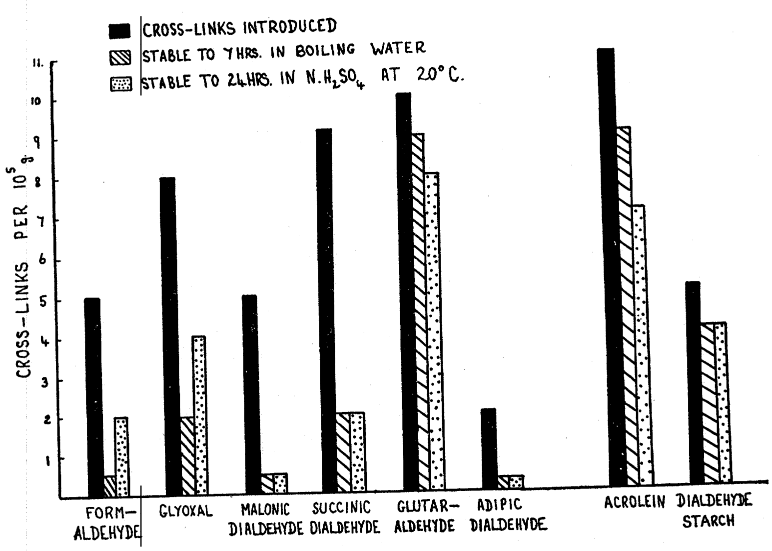


FIGURE 1.

Cross-linking with aldehydes.
Moles of cross-links introduced per 10^5g and their stability

results are included in Table I with results from similar treatments with the dialdehydes derived from malonic, succinic and adipic acids.

Relative stabilities of the cross-links introduced by the dialdehydes to the action of boiling water and dilute acid are represented in Figure 1 in the form of a histogram. For purposes of comparison formaldehyde and acrolein (see Part I) are also included in the diagram.

In view of the general superiority of glutaraldehyde over the other dialdehydes tested, the effects of time and temperature on its reaction with collagen have been examined together with the stability of glutaraldehyde cross-links to the action of warm aqueous alkali and to moist heat and perspiration.

Scarcity of tendon at the time when the time and temperature experiments were performed made it necessary to use a batch of wallaby tendons which, for an as yet unexplained reason, consistently gave somewhat low values for the number of cross-links introduced using a number of different cross-linking agents. However, some general conclusions can be drawn from the results given in Table II. At room temperature tanning is virtually complete in 24 hours and at 40° in about 16 hours. At pH 6.5, though hardly significantly at pH 8·0, the maximum number of cross-links introduced is increased by raising the temperature of reaction to 40°. Some additional twenty-four hour tannages in the range 20-40° were also carried out at pH 9 and 10. The numbers of cross-links per molecular weight unit of 10^5 were 8 and 9 respectively. From these results it is inferred that the increased reactivity of the basic groups of the collagen at higher pH values is offset by loss of aldehyde due to polymerisation.

TABLE II

Effect of Time and Temperature in Glutaraldehyde Treatments

Time (hours)	M_C		Cross-links per 10^5g	
	pH 6·5	pH 8·0	pH 6·5	pH 8·0
At 20° C				
2	16,300	12,500	3	4
8	15,300	9,700	3	5
24	8,000	6,600	6	8
48	8,400	5,500	6	9
At 40° C				
2	8,500	10,500	6	5
6	9,900	10,450	5	5
12	5,900	7,000	9	7
24	4,600	5,000	11	10

146 moles of glutaraldehyde per 10^5g collagen were offered.

Moist heat or moist heat in conjunction with the action of synthetic perspiration at pH 6·8 had little deleterious effect over the full 28-day period of the experiment (Table III). However, when tendons were similarly subjected to a mildly alkaline perspiration (pH 7·70) considerable diminution in the number of cross-links did occur in the latter half of the experimental period.

TABLE III
Further Stability Tests on Glutaraldehyde Treated Tendons

Treatment	Time days	Number of Cross-Links per 10^5g	
		4% aldehyde offered	12% aldehyde offered
Moist heat 40°C	13	6	9
100% r.h.	28	6	9
Perspiration at 40°C	13	5	7
pH 6·8	28	6	7
Perspiration at 40°C	13	6	7
pH 7·7	28	3	5
Saturated lime at 40°C	13	4	5
Control	—	6	9

As would be expected saturated aqueous lime at 40° proved to be more drastic in its action, causing a similar reduction in the measured number of cross-links in 13 days. In both alkaline treatments and much more markedly in the latter one, a reduction in the strength of the undenatured tendons was qualitatively observed suggesting that scissions in the peptide backbones may be an alternative explanation of the lower experimental results (see discussion).

(ii) Dialdehyde Starch

In Table IV the polymeric aldehyde, DAS is reacted with tendon for times ranging up to 72 hours, at 20°, pH 8. The number of cross-links increases steadily with time over this period. A further experiment has been done in which the treatment was at pH 6·0 for 72 hours at 20°. A mean result of 4 cross-links per 10^5g was obtained.

Stability measurements on the tendons prepared at pH 8·0 demonstrated that only a slight reduction in the number of cross-links resulted from 7 hours boiling in water or on treatment in dilute acid up to 120 hours.

(iii) Tetrakis (Hydroxymethyl) Phosphonium Chloride

The results of treating skin and tendon with THPC at 20°, pH 6·5 are given in Table V. Determination of the phosphorus content of the skin by wet oxidation to orthophosphate and estimation by the phospho - vanadio - molybdate method[7] gave 1·45%, representing an uptake of THPC of 8·7%. Good stability to boiling water and to dilute acid is demonstrated (see also Fig. 2).

TABLE IV
Effect of Time on Dialdehyde Starch Treatment at pH 8, 20°C

Time (hours)	M_c	Cross-links per 10^5g
4	19,900	2
24	14,700	3
48	11,400	4
72	10,300	5

50% by weight of the dialdehyde starch on the weight of the collagen was offered.

TABLE V

Tetrakis (Hydroxymethyl) Phosphonium Chloride at pH 6·5, 20°C

Time of treatment (hours)	Shrinkage temperature °C (skin)	Cross-links per 10^5g					
		Initial	Treatment in boiling water			Treatment in $N.H_2SO_4$ at 20°C	
			1 hour	4 hours	7 hours	16 hours	120 hours
2	79						
16	79	6	5	3	2	4	5

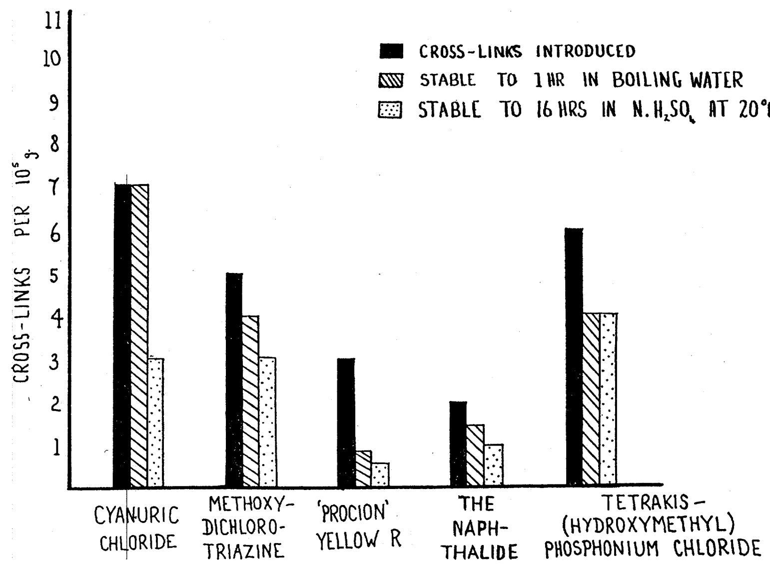


FIGURE 2.

Cross-linking with cyanuric chloride derivatives.
Moles of cross-links introduced per 10^5g and their stability

(iv) The Di-Isocyanates.

Results are given in Table VI for treatments with (a) HDI emulsified in water by the action of a detergent (b) di-isocyanates in pyridine (c) di-isocyanates in benezene in the presence of a tertiary amine and (d) water soluble di-isocyanate adducts.

In method (a) the number of cross-links introduced is small. "Teepol" 610, an anionic detergent, is slightly more effective than the non-ionic "Nonidet" P40 in assisting reaction. In processes (b) and (c) the air-dry tendon is reacted with the di-isocyanate in the presence of an organic tertiary base which in (b) is also the solvent for the di-isocyanate whereas in (c) benzene serves this function. Large weight increases ranging up to 100% on the weight of the tendon are obtained. Clearly these are due to polymerisation since estimates of the number of cross-links are generally low

TABLE VI

Cross-Linking with Di-isocyanates and their Derivatives

Compound	Treatment of Collagen	Uptake g/100g	M_c (tendon)	Number of cross-links 10^5g	Shrinkage temperature °C (skin)
(a) HDI	Emulsified in water "Nonidet" P40 pH6	low	49,000	1	66
HDI	Emulsified in water "Teepol" 610 pH7	low	25,000	2	63
(b) HDI	Pyridine 50°/4 hours	—	10,000	5	—
TDI	Pyridine 50°/4 hours	26†	66,000	1	68*
(c) HDI	Benzene + triethylamine 50°/4 hours	109†	45,000	1	87*
Potassium Bisulphite adduct of HDI	Water at pH5	0·6†	18,000	3	62
ditto of TDI	Water at pH5	2·2†	11,000	4	70
Succinimide adduct of HDI	Water at pH5	2·5†	19,000	3	65

* Shrinkages were very small even when heating extended to 100°

† Estimated from weight increase of small samples—must be regarded as approximate.

and extremely variable (see discussion). Shrinkage temperatures measured on skin similarly treated are also recorded. The temperature given is the point at which shrinkage was first observed; in some cases, however, the shrinkage was very small even when heating was extended to 100°C in water, or 110°C in glycerol water mixtures, which is suggestive of restriction due to polymer deposition rather than cross-linking.

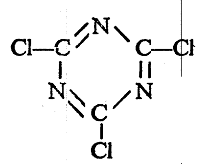
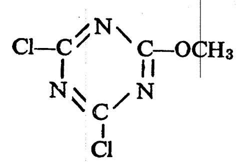
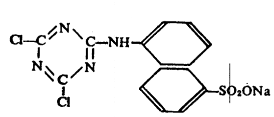
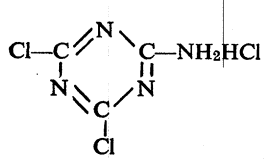
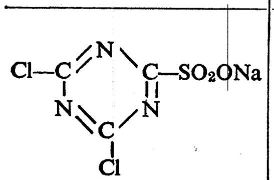
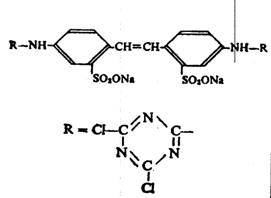
Since the performance of the di-isocyanates as cross-linking agents was disappointing, in (d) attention was turned to the addition compounds. It was hoped that these could be decomposed, releasing the free di-isocyanate, when in close proximity to reactive groups in the collagen, where conditions would favour the formation of a high proportion of monomeric bridges rather than reaction of the di-isocyanate molecules with each other. The expectation was that these compounds would be stable at room temperature but that on raising the temperaute the free compound would be released. In practice cross-linking occurred to a limited extent at room temperature with no evidence of concomitant polymerisation. Unfortunately raising the temperature did not increase the extent of cross-linking.

(v) Triazine Derivatives

Cyanuric chloride and a number of its derivatives have been evaluated as cross-linking agents for collagen. The results are given in Table VII.

Cyanuric chloride has the disadvantage of very low solubility in, yet high reactivity towards, water, leading to the formation of water insoluble and relatively inactive hydroxy-derivatives. The methoxy compound is only

TABLE VII
Cross-Linking by Cyanuric Chloride and Derivatives

Substance	pH of treatment	M_c (tendon)	Number of cross-links in 10^5g	Shrinkage temperature °C (skin)
	8·0	8,100	6	80
	9·0	7,660	7	83
	10·0	7,820	6	83
	8·0	8,060	6	77
	9·0	9,400	5	69
	7·0	13,100	4	63
	8·0	16,000	3	63
	9·0	33,300	1·5	
	10·0	133,000	0·5	
	7·0	12,500	4	
	8·0	7,350	7	65
	9·0	9,600	5	
	7·0	13,500	4	61
	8·0	9,500	5	61
	7·0	7,700	6	68
	8·0	11,500	4	68
	9·0	35,500	1·5	

slightly soluble but the reactivities of its two chlorine atoms are enhanced by the substituent group. Using aqueous acetone in the reaction mixture a moderate number of cross-links were introduced with both compounds. The amino derivative also introduced a similar degree of cross-linking at pH 8 but its effect on the shrinkage temperature of skin was less marked. Three compounds containing water solubilising sulphonate groups are also included, namely, the simple sulphonate, the naphthalide and the stilbene, but their performances were rather disappointing.

In Figure 2 the stabilities of the cross-links introduced by some of the above compounds and by Procion Yellow M-RS are shown.

Discussion

Although the lower aliphatic aldehydes are reactive towards collagen they are not effective stabilising agents except in cases where the compound reacts bifunctionally and in consequence is able to contribute to the formation of cross-links between adjacent polypeptide chains. The established tanning agent formaldehyde is an example of such a compound and more recently two dialdehydes, namely glyoxal and glutaraldehyde have been evaluated in various laboratory and works scale trials (see ref. (1) for discussion and six references to original work). Glyoxal, the first member of the series $OHC\text{-}(CH_2)_x\text{-}CHO$ where x=O, has not so far found wide application in the leather industry but glutaraldehyde (x=3) in the short time that it has been commercially available has shown great promise, not only as a complete tanning agent, but also in use with other established tanning materials. In Part I of this series stress-strain experiments on aldehyde tanned tendons demonstrated that in terms of the number of cross-links introduced and of their stability the order of efficiency as cross-linking agents was glutaraldehyde>glyoxal>formaldehyde. In the experimental section of the current paper similar methods are applied to the dialdehydes where x=1, 2 or 4 respectively. On the whole the performances of these aldehydes are not encouraging. Malonaldehyde (x=1) suffers from the disadvantages of instability and low solubility in water. Neither in terms of the number of cross-links introduced nor their stability does it offer any advantage over formaldehyde. Although succinaldehyde (x = 2) is more effective and approaches glutaraldehyde in the number of cross-links introduced, they are less stable to the action of boiling water and to dilute acid. On moving up the series beyond glutaraldehyde to adipaldehyde (x = 4) we again find a marked decrease in the efficiency of cross-linking.

As glutaraldehyde is the most efficient of the dialdehydes so far tested, the effects of varying time, temperature and pH are examined. However, no change in conditions increased the number of cross-links in tendon beyond the already reported maximum of 13 per mol. wt. unit of 10^5 or raised the shrinkage temperature of skin above 86°. Now it is generally considered that the aldehydes react with the amino groups of collagen and it is interesting to note in this connection that when de-aminated collagen is treated with glutaraldehyde, neither is the shrinkage temperature raised nor is there appreciable loss of aldehyde from the solution. In contrast the amino acid analysis of normal collagen treated with glutaraldehyde[1] shows a loss of lysine commensurate with the amount of aldehyde offered but otherwise little change in composition. If one supposes that all the lysine reacted with the aldehyde to yield cross-links the number would be about 16 per mol. wt. unit of 10^5. It seems, therefore, that a minority of the lysine side-chains are unable to participate, presumably for steric reasons.

The differing behaviours of the x = 2, 3 and 4 dialdehydes suggest that single dialdehyde molecules do not form simple bridges between pairs of polypeptide chains. In some recent experiments using ^{14}C-glutaraldehyde the total number of aldehyde molecules fixed was found capable of exceeding a one to one equivalence with the primary amino-groups[1], suggesting that each cross-link involved at least two molecules of the aldehyde. Aso and Aito[8] and Meyersen, Schulz and Kern[9] have recently inferred, mainly from spectral data, that glutaraldehyde polymerises as a chain of six membered pyranose rings joined by ether linkages. It is possible that similar types of structures occur in the glutaraldehyde cross-link. With the neighbouring dialdehydes the analogous rings would presumably have to be formed from a different number of atoms, which may achieve cyclisation less readily, or when formed such rings and hence the ligand, may be inherently less stable.

Laboratory tests have shown that glutaraldehyde tanned leather has good resistance to the action of moisture, heat and perspiration[10, 11]. With glutaraldehyde tanned tendons the number of cross-links is substantially unchanged by warm moist conditions alone or by a warm neutral perspiration solution over considerable period of time (28 days). Alkaline perspiration causes some reduction in the measured number of cross-links in the latter stage of this rather drastic test. However, when the tendons are subjected to caustic alkalinity of saturated calcium hydroxide at 40° a similar diminution occurs at about twice the rate and is accompanied by a more obvious deterioration in mechanical strength. It is possible that under alkaline conditions peptide bond scission rather than rupture of cross-links is responsible for the lower number of determined cross-links. On denaturation the random network would consequently deviate more widely from the idealised model of the Flory-Rehner equation[12].

Turning to the polymeric aldehyde DAS, cross-links of good stability are introduced although the initial measured number is not high. In assessing the results obtained with this compound two possible complications arise. Firstly the uptake of DAS is fairly high[13] and the estimate of the number of cross-links would be materially increased if based on the collagen weight. Secondly the random network of the test specimen is polymerically heterogeneous and may, therefore, give misleading results.

Filachione[14] has shown that treatment with THPC yields a leather of natural colour and satisfactory shrinkage temperature. The cross-links introduced are probably formed by reaction of the hydroxmethyl groups with the lysine side chains of the collagen. They would, therefore, be expected to resemble in some degree those introduced by formaldehyde. In reality they have been shown to be more stable. Such increased strength may be due to the tetra functionality of the THPC molecule. On the basis of phosphorus content it is likely that more than one THPC molecule is involved in each cross-link.

Three methods are described in which cross-linking is attempted by reacting collagen with di-isocyanates. The low solubility in water of these compounds and yet their ready polymerisation under its agency present considerable experimental difficulties. However, there is also evidence that little or no reaction occurs when water is rigorously excluded. Reaction of HDI and TDI with air-dry collagen in organic solvents and in the presence of organic bases leads apparently to cross-linking but, as was suggested in connection with DAS, the network formed on denaturation is heterogeneous due to deposited polymer. Experiments on the hydrothermal shrinkage of similarly treated skin suggest that the actual process of denaturation may be mechanically obstructed by the deposited polymer, and consequently the stress-strain results should be treated with reserve.

By using water soluble addition compounds of the di-isocyanates which break down under the influence of reactive groups in the collagen giving the free di-isocyanate, cross-linking with little simultaneous polymerisation is obtained although the degree of cross-linking is not high.

Finally the cross-linking action of some chlorotriazine derivatives is considered. Substitution of the first chlorine atom of cyanuric chloride, the parent compound, readily takes place but the second and third are progressively less reactive, moreover, their reactivities are influenced by the substitutions already made. Thus a methoxy group is stated to enhance and an amino group to retard reaction[15]. However, in the cross-linking of collagen about the same maximum numbers of cross-links are obtained with each compound. It is generally considered that the chlorotriazines react mainly with the primary amino groups of collagen[16] and consequently the second chlorine atom which must react to complete the formation of the cross-link tends to be de-activated. An alternative possible explanation is that much of the methoxy-derivative is lost due to preferential reaction with the water.

Cyanuric chloride itself is almost insoluble in water but when reacted with collagen in aqueous acetone is among the more efficient members of this group providing that penetration can be achieved before reaction with the water leading to formation of the insoluble, inactive mono-hydroxy compound.

When the first chlorine atom of cyanuric chloride is replaced by a conventional dye molecule residue, a reactive dye-stuff is formed. Water solubilising groups attached to the dye side chain confer solubility on the molecule as a whole and chemical reaction with the substrate is by substitution of one or both of the remaining chlorine atoms on the triazine nucleus. The successful "Procion" range of reactive dyestuffs are of this type. Preliminary experiments using the "Procion" dyes on tendon showed that they were not very effective as cross-linking agents. For this reason the simple though sparingly water soluble sulphonate derivative was prepared and the more soluble naphthalide sulphonate obtained. The performance of both compounds were rather disappointing, possibly due to their bulky side chains and consequent slow penetration with resultant loss of their more reactive chlorine atoms to the water.

Rather surprisingly the stilbene derivative with two dichlorotriazine residues is only slightly more efficient than the mono-triazines. This, too, may be due to steric hindrance.

The stability of the triazine cross-link appears to be quite good towards boiling water but some sensitivity to dilute acid is shown. Although under the best conditions the aldehydes introduce nearly twice as many cross-links as the triazines yet the effect of the smaller number of triazine cross-links on the shrinkage temperature of skin can be nearly as great.

Of the compounds tested in this series the bifunctional aldehydes glutaraldehyde and acrolein appear to be the most promising both with respect to the number of cross-links introduced and to their stability. Cyanuric chloride and some of its derivatives are also relatively efficient but in view of the low activity of the last chlorine atom to be substituted it seems likely that these compounds will prove more useful for attaching other compounds chemically to collagen, *e.g.* dyes, than as cross-linking agents in themselves.

During the course of these investigations confidence in the stress-strain method has increased. Whilst it may sometimes be questioned whether the estimate of the number of cross-links is true in an absolute sense, nevertheless the method enables useful comparisons to be made between compounds which are fairly similar in chemical constitution.

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